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**14. ABSTRACT**

In eukaryotes, homologous recombination and the homology-directed repair of DNA double-strand breaks are mediated by the RAD51 recombinase. In catalyzing recombination reactions, RAD51 must first form a right-handed helical filament, termed the presynaptic filament, on single-stranded DNA. Emerging evidence indicates that BRCA2 acts a recombination mediator by promoting the assembly of the RAD51 presynaptic filament. BRCA2 binds DNA and associates with RAD51. Our laboratory has established biochemical systems to examine the recombination mediator function of BRCA2. The main focus of my fellowship project is to define the role of DNA binding in this BRCA2 function. The BRCA2 DNA-binding domain (DBD) represents a highly conserved region within BRCA2-like molecules and harbors a significant portion of tumor-derived missense mutations, underscoring the importance of addressing the functional significance of this BRCA2 domain.

**15. SUBJECT TERMS**
DNA Repair; Tumor Suppressor

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## Table of Contents

## Introduction

Germ line mutations in the breast cancer susceptibility gene BRCA2 predispose carriers to early-onset breast cancer. BRCA2-deficient cells exhibit chromosomal instability and increased sensitivity to genotoxic agents. The involvement of BRCA2 in DNA double-strand break repair through the homologous recombination pathway is likely to account for these phenotypic changes, however, the mechanistic role of BRCA2 in homologous recombination remains to be defined. The main goal of this fellowship project is to define the role of DNA binding in recombination mediator function of BRCA2. Moreover, cancer-associated mutations found in the DNA-binding domain (DBD) of BRCA2 will be assessed, so as to rationalize how mutations in BRCA2 lead to genome instability and breast cancer.

## Body

To elucidate the relevance of DNA-binding by BRCA2 in the DNA homology-directed repair of chromosomal breaks and to delineate the effect of cancer mutations on this BRCA2 function, I conducted the following molecular studies as outlined in the Statement of Work of my fellowship proposal.

To delineate the mode and the specificity of DNA binding by BRCA2, I purified human BRCA2 DBD and defined its DNA binding properties. I examined the affinity of BRCA2 DBD for different length DNA substrates. I conducted DNA binding with purified BRCA2 DBD. In these experiments, $^{32}$P-labeled ss DNA substrates free of secondary structure (e.g. poly dT) were individually incubated with several different amounts of BRCA2 DBD, followed by analysis of the reaction mixtures in a non-denaturing polyacrylamide gel. After drying, the gel was subject to phosphorimaging analysis to detect DNA binding. The results indicate that the minimal DNA binding region of BRCA2 DBD is 24 nucelotides (Figure 1 in the Appendices). Studies conducted in the Pavletich laboratory (Yang et al, 2002, 2005) have found that the mouse BRCA2-DBD and *Ustilago maydis* Brh2 protein (the BRCA2 orthologue) both bind preferentially to a duplex-ssDNA junction that harbors a 3' ss overhang. Thus, it will be of interest to investigate whether human BRCA2 DBD likewise possesses specificity for this DNA structure and additional DNA substrates that resemble HR intermediates (partial duplex, D-loop, etc.)

BRCA2 contains three canonical OB (oligonucleotide and oligosaccharide binding) folds that confer a DNA binding ability. Due to the exceedingly large size of human BRCA2 (3,418 amino acid residues), it has not yet been possible to obtain sufficient amounts of full-length protein for mechanistic studies. Thus, our research group employed a modular approach that entails combining selected BRC repeats that are known to bind RAD51 with avidity (Chen et al, 1998; Wong et al, 1997), with the DBD of BRCA2 (SanFilippo et. al., 2006). To determine the importance of OB folds in DNA binding affinity by BRCA2, I designed constructs consisting of a RAD51 binding module (BRC4) fused to either individual OB folds or OB folds in tandem. I expressed these polypeptides fused to a sixhistidine tag in bacteria. I carried out a five-step procedure - encompassing ammonium sulfate precipitation of bacterial extract, an affinity step in

nickel-NTA agarose, and also chromatographic fractionation in macro-hydroxyapatite (MHAP) column to purify BRCA2 DBD variants. Although the purification scheme devised is optimal for these mutant polypeptides, the bacterial extract does not present the most suitable system for purification of BRCA2 DBD variants. Recently, a postdoctoral fellow in our laboratory successfully carried out expression and purification of BRCA2 BRC4 DBD in insect cell system. To determine the importance of OB folds in DNA binding affinity by BRCA2, I designed constructs consisting of a RAD51 binding module (BRC4) fused to either individual OB folds or OB folds in tandem together with the C-terminal region of BRCA2 (CTRB). Thus, I am currently focusing on expression and purification of the BRCA2 BRC4 DBD CTRB variants to near homogeneity in insect cells. I expressed these polypeptides fused to GST and six-histidine tags. Co-expression of BRCA2 DBD constructs with BRCA2-associated protein, Dss1, increased the solubility of the BRCA2 DBD variants. Since Dss1 binds OB1 of BRCA2, I was able to co-express BRC4 OB1 CTRB and BRC4 OB12 CTRB with Dss1 in order to enhance the solubility of these polypeptides. To this date, I successfully purified BRC4 OB1 CTRB, BRC4 OB12 CTRB, and BRC4 OB23 CTRB variants (Figure 2 in the Appendices). I expect to purify BRCA2 BRC4 OB2 CTRB and BRC4 OB3 CTRB variants and to assay the resulting polypeptides for DNA binding, alongside the wild type BRC4 DBD CTRB, in the electrophoretic DNA mobility shift assay within the next three months. I will also test the resulting polypeptides in *in vitro* homologous pairing reaction to determine the relevance of OB folds on recombination mediator function of BRCA2.

A striking feature of the BRCA2 DBD is the Tower anchored on OB2. The Apex of the Tower is a three helix bundle that resembles the helix-turn-helix double-stranded DNA binding motif. We have shown that BRCA2 DBD is capable of binding double-stranded DNA. To investigate the contributions of the Tower domain to the DNA-binding activity of BRCA2, I expressed and purified constructs deleted for the Tower (delT) or the Apex of the Tower (delA) in the context of the BRCA2 BRC12 DBD CTRB. I carried out DNA binding experiments with the resulting polypeptides and observed a reduced binding affinity for double-stranded DNA by the polypeptide deleted for the Tower domain when compared to its wild type counterpart (Figure 3C and 3D in the Appendices). The preliminary results demonstrate that the deletion of the Apex of the Tower also attenuates dsDNA binding activity of BRCA2 BRC12 DBD CTRB.

The importance of the Tower domain of BRCA2 will be examined in recombination mediator assay. I expect to find that attenuation of DNA binding affinity through deletion of OB folds and the 3HB of the Tower domain will have a significant impact on the recombination mediator function of BRC4 DBD CTRB.

The BRCA2 DBD represents a highly conserved region within BRCA2 orthologues and harbors a significant portion of cancer-derived missense mutations, emphasizing the importance of this region in the tumor suppressor function of BRCA2 (BIC database: Szabo et al, 2000). To examine the effect of tumor-derived DBD mutations on BRCA2-dependent recombination, I introduced the cancer-associated Tower mutations - E2856A, I2944F, K2950N, and A2951V – into BRC3/4-DBD. I will express and purify the mutant variants, and delineate their DNA binding and recombination mediator activities.

The functional consequence of the cancer-associated OB2 mutations – S2988G and Q3026E – will be similarly assessed. The results will provide important molecular information to link BRCA2 mutations to the cancer phenotype.

## Key Research Accomplishments

**Task 1: To delineate the mode and the specificity of DNA binding by BRCA2.**

- Defined the minimal DNA length required for BRCA2 binding
- Fused BRC4 region to either individual OB folds or OB folds in tandem and CTRB domain for expression and purification in insect cell system
- Devised purification scheme for the aforementioned BRCA2 BRC4 DBD CTRB mutant polypeptides and identified insect cells to be optimal for expression and purification of BRCA2 DBD variants to near homogeneity
- Purified BRCA2 constructs deleted for the Apex of the Tower domain (delA) or the Tower domain (delT) in the context of the BRCA2 BRC12 DBD CTRB and carried out DNA binding experiments with these polypeptides
- Introduced the cancer-associated Tower mutations - E2856A, I2944F, K2950N, and A2951V - into BRC4 DBD constructs

**Task 2: Define the relevance of the OB folds and the Tower domain on the recombination mediator function of BRCA2 and examine the effect of tumor-derived DBD mutations on this BRCA2 function.**

- Purified human RAD51 and RPA proteins and confirmed their activity in recombination mediator assays previously established in the laboratory
- Carried out recombination mediator assays using the wild type BRC4-DBD polypeptide in order to optimize the reaction conditions for future assessment of BRC4 DBD CTRBvariants

## Reportable Outcomes

Yale University provides an excellent training environment for breast cancer research, with a formal Breast Cancer Research Program (BCRP) within the Yale Cancer Center, a NCI-designated Comprehensive Cancer Center. As a component of my training, I attend monthly meetings of the BCRP and present my research findings in the spring semester. This summer I presented my poster at the Era of Hope Meeting in Baltimore, MD (June 25-28, 2008).

## Conclusion

The focus of my research is to delineate the importance of DNA binding by BRCA2 in its recombination mediator function. By characterizing the biochemical functions of BRCA2, we should gain a molecular understanding of why breast

tumorigenesis is associated with BRCA2 mutations. The knowledge garnered from my studies could very well be exploited for the prevention, diagnosis, and treatment of breast cancer. The experimental systems that I will devise while conducting my research studies will be a valuable tool for assessing the functional consequence of BRCA2 mutations in breast cancer patients.

**References:**

Chen PL, Chen CF, Chen Y, Xiao J, Sharp ZD, Lee WH. The BRC repeats in BRCA2 are critical for RAD51 binding and resistance to methyl methanesulfonate treatment. Proc Natl Acad Sci U S A. 1998 Apr 28;95(9):5287-92.

Chen CF, Chen PL, Zhong Q, Sharp ZD, Lee WH. Expression of BRC repeats in breast cancer cells disrupts the BRCA2-Rad51 complex and leads to radiation hypersensitivity and loss of G(2)/M checkpoint control. J Biol Chem. 1999 Nov 12;274(46):32931-5.

Jasin M. Homologous repair of DNA damage and tumorigenesis: the BRCA connection. Oncogene. 2002 Dec 16;21(58):8981-93. Review.

Kowalczykowski SC. Molecular mimicry connects BRCA2 to Rad51 and recombinational DNA repair. Nat Struct Biol. 2002 Dec;9(12):897-9.

San Filippo J, Chi P, Sehorn MG, Etchin J, Krejci L, Sung P. Recombination Mediator and Rad51 Targeting Activities of a Human BRCA2 Polypeptide. J Biol Chem. 2006 Apr 28;281(17):11649-57.

Sung P. Catalysis of ATP-dependent homologous DNA pairing and strand exchange by yeast RAD51 protein. Science. 1994 Aug 26;265(5176):1241-3.

Sung P. Mediating repair. Nat Struct Mol Biol. 2005 Mar;12(3):213-4.

Sung P, Krejci L, Van Komen S, Sehorn MG. Rad51 recombinase and recombination mediators. J Biol Chem. 2003 Oct 31;278(44):42729-32. Epub 2003 Aug 11. Review.

Szabo C, Masiello A, Ryan JF, Brody LC. The breast cancer information core: database design, structure, and scope. Hum Mutat. 2000;16(2):123-31.

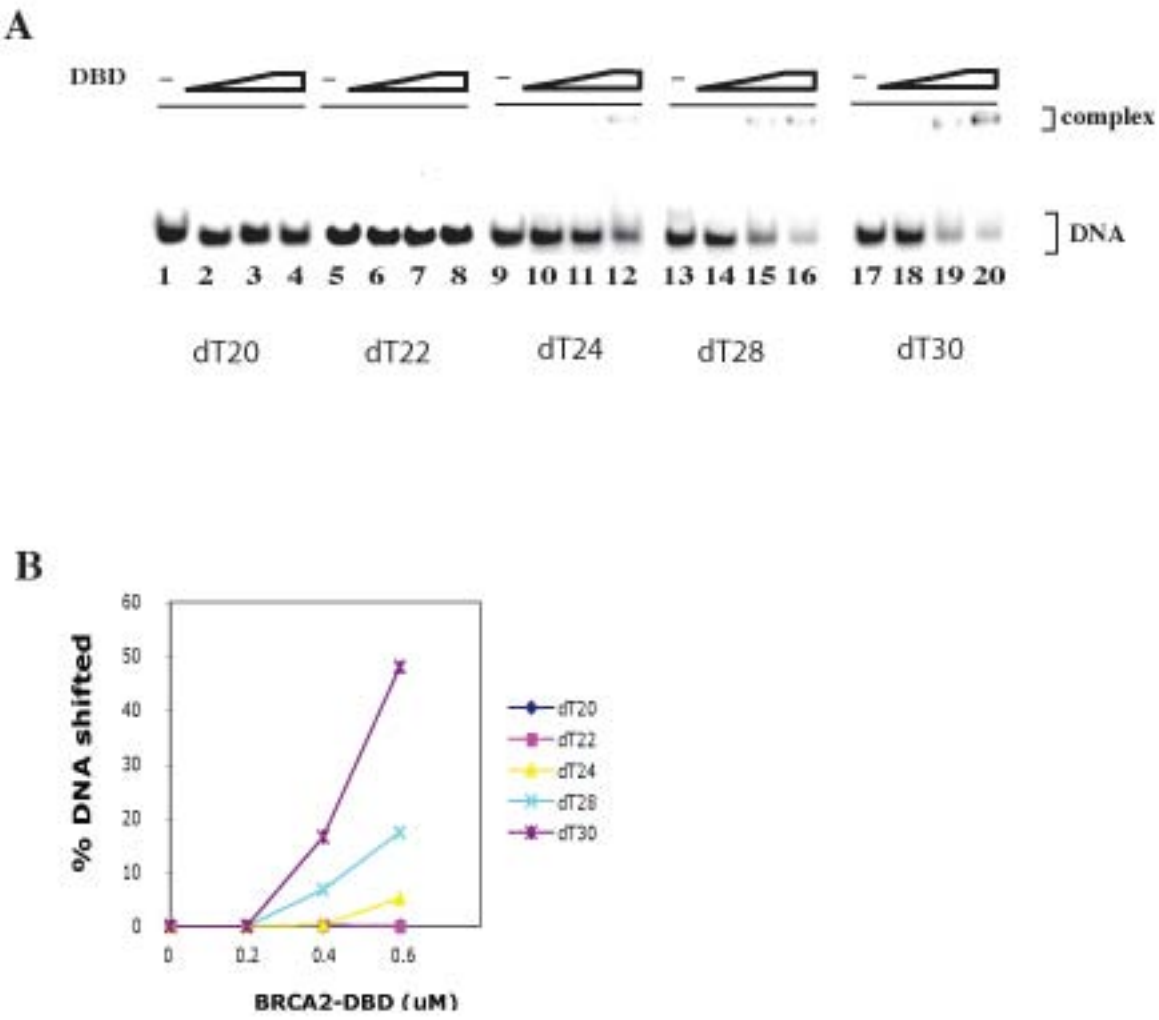
Wong AK, Pero R, Ormonde PA, Tavtigian SV, Bartel PL. RAD51 interacts with the evolutionarily conserved BRC motifs in the human breast cancer susceptibility gene BRCA2. J Biol Chem. 1997 Dec 19;272(51):31941-4.

Xia F, Taghian DG, DeFrank JS, Zeng ZC, Willers H, Iliakis G, Powell SN. Deficiency of human BRCA2 leads to impaired homologous recombination but maintains normal nonhomologous end joining. Proc Natl Acad Sci U S A. 2001 Jul 17;98(15):8644-9.

Yang H, Jeffrey PD, Miller J, Kinnucan E, Sun Y, Thoma NH, Zheng N, Chen PL, Lee WH, Pavletich NP. BRCA2 function in DNA binding and recombination from a BRCA2-DSS1-ssDNA structure. Science. 2002 Sep 13;297(5588):1837-48.

Yang H, Li Q, Fan J, Holloman WK, Pavletich NP. The BRCA2 homologue Brh2 nucleates RAD51 filament formation at a dsDNA-ssDNA junction. Nature. 2005 Feb 10;433(7026):653-7.

## Figure 1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DBD
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DNA
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dT20
dT22
dT24
dT28
dT30
B
% DNA shifted
60
50
40
30
20
10
0
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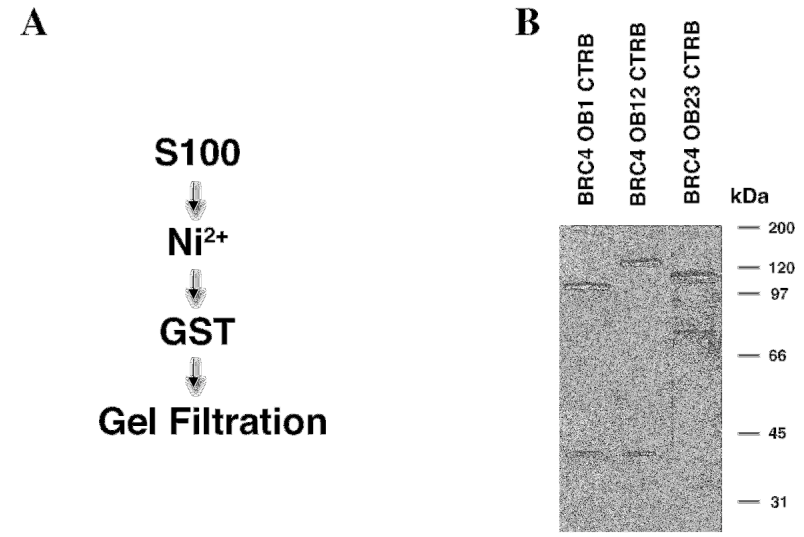
0.6
BRCA2-DBD (uM)
dT20
dT22
dT24
dT28
dT30

Figure 2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S100
Ni2+
GST
Gel Filtration
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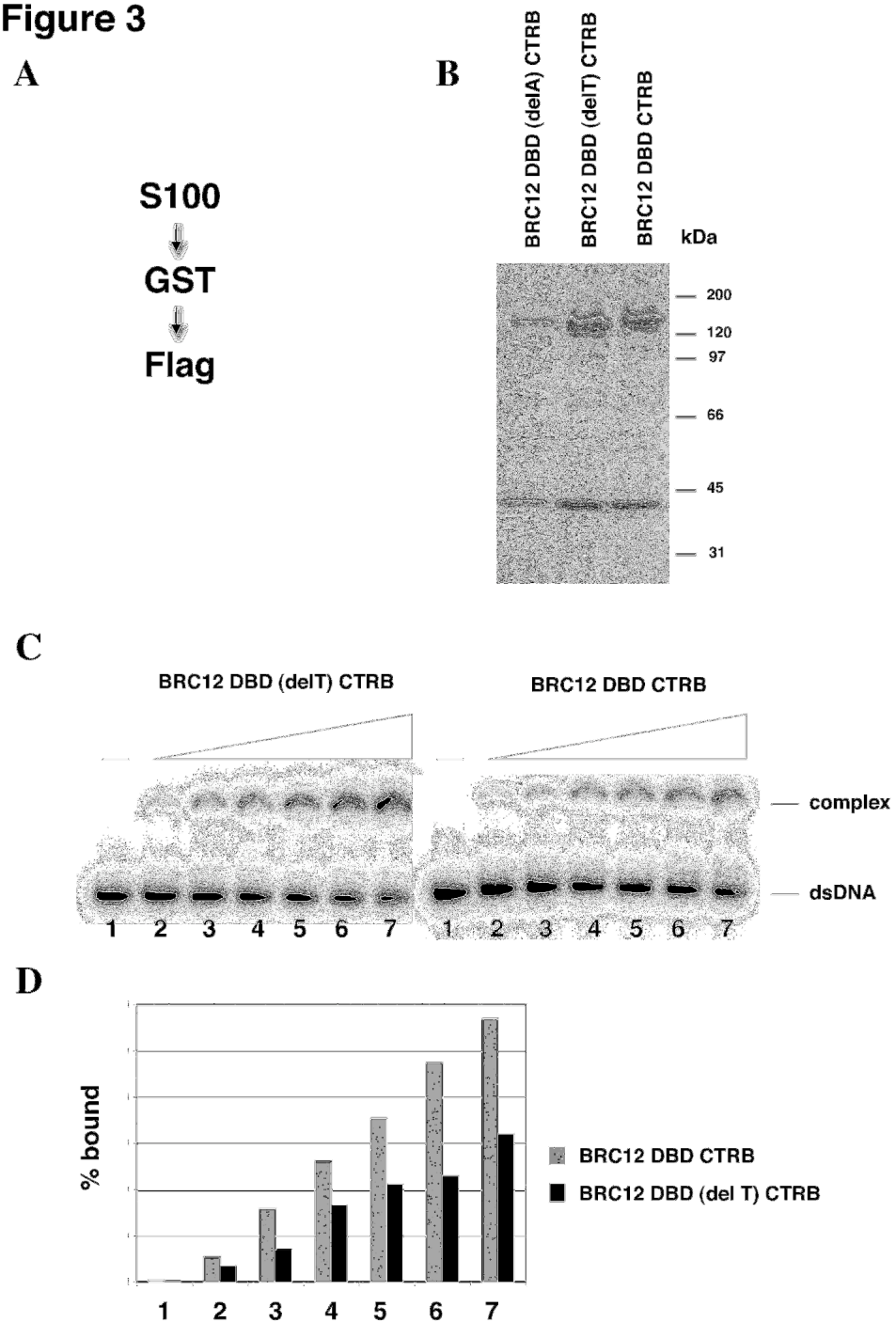
BRC4 OB12 CTRB
BRC4 OB23 CTRB
kDa
200
120
97
66
45
31

Figure 3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S100
GST
Flag
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BRC12 DBD (delA) CTRB
BRC12 DBD (delT) CTRB
BRC12 DBD CTRB
kDa
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97
66
45
31
C
BRC12 DBD (delT) CTRB
BRC12 DBD CTRB
complex
dsDNA
1 2 3 4 5 6 7
1 2 3 4 5 6 7
D
% bound
1 2 3 4 5 6 7
BRC12 DBD CTRB
BRC12 DBD (del T) CTRB

**Figure 1. The minimum DNA length required for BRCA2 binding is 24 nucleotides. (A)** Purified BRCA2-DBD (200 to 600 nM in lanes 2 to 4, 6 to 8, 10 to 12, 14 to 16, and 18 to 20) was examined for its ability to bind $^{32}$P-labeled 20, 22, 24, 28, and 30 dT DNA. **(B)** The results in **A** are graphed.

**Figure 2. Purification of OB fold variants within the context of BRC4 and CTRB domains. (A)** Chromatographic procedure designed for purification of OB fold construct variants. **(B)** Purified BRC4 OB1 CTRB, BRC4 OB12 CTRB, and BRC4 OB23 CTRB were analyzed by SDS-PAGE. BRC4 OB1 CTRB and BRC4 OB12 CTRB were co-purified with Dss1 (as described in the body of the report).

**Figure 3. Deletion of the Tower domain attenuates dsDNA binding activity of BRC12-DBD-CTRB. (A)** Purification scheme for BRC12 DBD CTRB, BRC12 DBD (delA) CTRB, and BRC12-DBD (delT) CTRB polypeptides. **(B)** Purified BRC12 DBD CTRB, BRC12 (delA) CTRB, and BRC12- BD (delT) CTRB were analyzed by SDS-PAGE. These polypeptides were co-purified with Dss1 (as described in the body of the text). **(C)** Purified BRC12 DBD CTRB and BRC12 DBD (delT) CTRB (150 to 900 nM in lanes 2 to 7) were incubated with 30nM $^{32}$P-labeled dsDNA at 37$^{o}$C for 5min. The reaction mixtures were analyzed by electrophoretic mobility shift assay. **(D)** The results in **C** are graphed.